INTRODUCTION

Breast cancer, a hormone-dependent cancer, is an urgent public health problem in Western countries and is becoming an increasingly urgent problem in Asian ones (Parkin, 2001; Smigal et al., 2006). The incidence in Europe, North America and Oceania is greater than 100 per 100,000. Despite the increase in recent years, the incidence remains below 30 per 100,000 in Asian countries such as China and Japan (Key et al., 2001). The studies of geographical variation, time trends and populations migrating from Eastern countries to Western ones suggest an important role of the environmental and dietary factors in the etiology of breast cancer (Ziegler et al., 1993).

Most studies investigated the relation between breast cancer risk and diets, including meats, fruits, vegetables, soybean and milk (Ahn et al., 2005; Wakai et al., 2005; Trock et al., 2006). Vegetables and fruits contain numerous bioactive and potentially anticarcinogenic substances including carotenes, dithiolthiones, flavoids, indoles, isothiocyanates, phenols, folic acid and vitamins C and E. There are many possible mechanisms by which the above substances might inhibit carcinogenesis, such as antioxidant effects, increases in cell-to-cell communication, activation of enzymes involved in carcinogen detoxification, alteration of estrogen metabolism, effects on DNA methylation and repair and anti-proliferative effects (Shrubsole et al., 2001). The blue-green microalga Spirulina platensis, used in daily diets of natives in Africa and America, were found to be a rich natural source of proteins, carotenoids, and other micronutrients. Experimental studies in animal models demonstrated an inhibitory effect of Spirulina on oral carcinogenesis (Mathew et al., 1995).

Other reports showed that Spirulina displays a preventive action on fatty liver induced in mouse and rat by the administration of a 60% fructose diet, or carbon tetrachloride and decrease the cancer risk in rats (Rodriguez-Hernández et al., 2001). So, the aim of the present work was to investigate the probable therapeutic effects of Spirulina platensis on induced mammary tumour in rats with Estrogen and Ki67 expression by immunostaining technique.

MATERIAL AND METHODS

Animals and tumor induction

Sixty female Sprague-Dawley rats inbred in animal house of Zoology Department, Faculty of Science, Mansoura University were used. Animals were maintained in groups of 5 or 6 per cage with feed and...
water *ad libitum* and artificially lighted 12 h/day. At 50 days of age, rats were segregated into four groups, 15 rats each according to the treatment regimen as follows, (1) Control, (2) Intrapertitoneal (i.p.) injections of 35 mg/kg single dose of dimethylbenz (a) anthractene (DMBA) dissolved in peanut oil, (3) Intrapertitoneal (i.p.) injections of 35 mg/kg single dose of (DMBA) with feeding the animals on 5% *Spirulina* which mixed with the standard diet throughout the experimental period and (4) 5% *Spirulina* fed group. At the end of six month of rat age, the animals were sacrificed.

**Histological and immunohistochemical preparations:**

All mammary tissues were removed and the specimens (3-5 mm thick) were fixed in 10% neutral formalin and processed to be paraffin blocks. Sections of 3 µm were prepared and stained with hematoxylin and eosin for microscopic examination and other for determination of proliferation marker (Ki67) and Estrogen by immunostaining technique. Some sections were mounted on Histogrip (Zymed, USA) coated glass slides and air-dried overnight at room temperature. Immuno-histochemical staining was performed using an avidin–biotin peroxidase complex. Briefly, samples were treated with 0.6% hydrogen peroxide in methanol for 30min to block endogenous peroxidase activity. Staining of formalin-fixed tissues requires boiling tissue sections in 10mM citrate buffer, pH 6.0, (Neomarkers Cat. #AP-9003) for 20 min which was followed by cooling at room temperature for 20 min. The slides were incubated with normal goat serum (1:10) (Neomarkers, USA) for 10 min and then with mouse monoclonal Ki67 and Estrogen antibodies (Neomarkers, USA), at dilution of 0.5-1.0µg/ml for 60 min at room temperature. The sections were further incubated with biotinylated goat anti-rabbit IgG diluted to 1:500 (Sigma–Aldrich, St. Louis, Missouri, USA) coated glass slides and air-dried overnight at room temperature. Immuno-histochemical staining was performed using an avidin–biotin peroxidase complex. Briefly, samples were treated with 0.6% hydrogen peroxide in methanol for 30min to block endogenous peroxidase activity. Staining of formalin-fixed tissues requires boiling tissue sections in 10mM citrate buffer, pH 6.0, (Neomarkers Cat. #AP-9003) for 20 min which was followed by cooling at room temperature for 20 min. The slides were incubated with normal goat serum (1:10) (Neomarkers, USA) for 10 min and then with mouse monoclonal Ki67 and Estrogen antibodies (Neomarkers, USA), at dilution of 0.5-1.0µg/ml for 60 min at room temperature. The sections were further incubated with biotinylated goat anti-rabbit IgG diluted to 1:500 (Sigma–Aldrich, St. Louis, Missouri, USA) for 10 min, followed by incubation with peroxidase-conjugated streptavidin diluted to 1:3000 in phosphate-buffered saline for 15 min. The peroxidase reaction was performed using 0.02% 3, 3-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide and counterstaining was performed with hematoxylin for 1min. In case of negative control, the primary antibody was omitted. The positive stains are brown nuclear stain and the count stain is haematoxylin.

Each section was counted manually at high power (X400) after identifying at low power (x100) the representative areas with the highest concentration of stained cells according to the recommendation of Cohen et al. (1993) to count the labelling indices of Ki67 and estrogen expression.

About 1000 cells/slide were counted in each of five microscopic fields from well-labelled areas to determine the average of Ki67 and Estrogen Labelling index (LI). LI was expressed as a percentage of labelled cells (positive for immunostaining reaction) of the total number of cells counted in each specimen. All identifiable staining was regarded as positive. The results are expressed as mean plus or minus standard deviation (LI = mean ± SD %).

**RESULTS**

**Histopathological results:**

(a) Control group

Figure 1a shows most of a breast lobule at a low magnification. The branch duct system is surrounded by relatively dense fibrous interlobular tissue at the periphery of which is adipose tissue. The duct system is lined with cuboidal or low columnar epithelial cells with oval nuclei. The larger ducts often have two layers of cells, whereas the smaller ducts have only a single layer of cells.

(b) *Spirulina*-treated animals

Breast of *Spirulina*-treated animals showed that the overall structure of the breast is much like that of the control animals (Fig. 1b). This is in agreement with earlier studies, that no significant differences between control and *Spirulina* treated animals in food intake, growth rate or carbon dioxide production. All animals remained apparently healthy, and had similar organ weights. These studies suggested that spirulina may be safely used as a supplemented source of proteins, vitamins and minerals in rat diets (Toshi et al., 1991; Tranquille et al., 1994; Narsinga, 1996).

c) DMBA precursors treated animals

In DMBA group, all the animals have ductal breast carcinoma and 70% of them displayed invasive tumour. Figure 1 c&d shows that ductal carcinoma is divided into invasive and in situ types depending on whether the malignant cells have branched basement membrane of the duct and invaded the stroma. In invasive ductal carcinoma, we showed the mitotic and necrosis figures, also the invasion of the tumour were present. The invading epithelial cells formed small ductal structures, solid nests, and even solid sheets of cells. The stroma was frequently very fibrotic.

d) DMBA precursors and *Spirulina*-treated animals

In this group, the carcinoma in situ of breast was represented in two rats 2/15) and 13/15 had no malignant, but as shown in figure 1e, there is dilation of ducts that formed cysts in which displayed by apocrine hyperplasia and metaplasia.

Proliferating cell nuclear antigen (Ki67) was exclusively nuclear and of variable intensity (figure 2a). The labelling index of Ki67 in control group was 20±5. A large range of proliferating cells in each ductal structure varied from 0 to 25%. Also, *Spirulina*-treated animals showed the overall appearance of the breast was much like that of the control animals (Fig. 2b).

The strong positive and highly labeled index (85±10) of Ki67 (the positive rang was from 40% to 95%) was present in DMBA precursors treated animals where the tumor growth was accompanied with high proliferation. In DMBA precursors and *Spirulina* treated animals, the proliferation decreased and the labeling index of Ki67 was 25±5 and the positive range was from 15 to 30.

On the other hand, experimental studies on rodent mammary tissue have demonstrated hormones as endogenous factors that influence mammary carcinogenesis; estrogens have attracted the most
Fig. 1. Photomicrographs of breast sections from female rats (A) the normal breast shows adipose tissue. (B) the breast of *Spirulina* treated animals. (C) the breast carcinoma with High proliferation and the lumen of duct fill with epithelial cells. (D) invasive breast cancer with mitotic figures M, necrosis N, fibrotic stroma S and malignant epithelial cells form small ductal structures D. (E) DMBA and *Spirulina* treated animals show the similar feature as in normal with some differented, the dilation of ducts form cysts C, fibrosis F and apocrine metaplasia AP. H&E X 250 original magnification.

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On the other hand, experimental studies on rodent mammary tissue have demonstrated hormones as endogenous factors that influence mammary carcinogenesis; estrogens have attracted the most attention. The ER was positive in 13 of 15 of DMBA group, but groups 1, 2 and 4 have the similar estrogen (ER) immunostaining results as the following; the estrogen was nuclear staining as shown in figure 3. The mean ± SD In control group the ER labeling index was 35±6 where the range of ER expression extended from 20 to 41% but in DMBA group the ER labeling index was 55±8 where the positive range from 0 to 63% (2 of 15 were negative for ER). In DMBA *Spirulina platensis* treated group, the range of ER expression varied from 25 to 45% with labeling index 40±5.

**Immunohistochemical results:**

Proliferating cell nuclear antigen (Ki67) was exclusively nuclear and of variable intensity (Fig. 2a). The labelling index of Ki67 in control group was 20±5. A large range of proliferating cells in each ductal structure varied from 0 to 25%. Also, *Spirulina*-treated animals showed the overall appearance of the breast was much like that of the control animals (Fig. 2b).

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Also, the present work is in agreement with that of cancer. Premkumar et al. (2004) found that Spirulina given orally to mice inhibited the induced genotoxicity by cisplatin and urethane. Recent studies revealed that proliferating cell nuclear antigen (PCNA) and ER indices were significantly correlated, and that an apparent correlation between PCNA and Ki67 indices was close to significance (Pena et al., 1998; Nieto et al., 2000); the Ki67 index was significantly correlated with ER (P<0.01). Such findings are in accord with findings in human breast cancer, indicating that well-differentiated tumours maintain some hormone regulatory mechanisms and are associated with a relatively low proliferative rate. Immunoreactive products of ER in normal and neoplastic mammary glands were localized in the nuclear area of cells but varied in respect of the number of positive cells and the intensity of the reaction product. Similar results were reported for human breast cancer and may be attributed to differentiated cell activity; ER content has also been related to tumour histopathology and tumour growth fraction in human beings (Raymond and Leong, 1989; Snead et al., 1993).

The present results suggested that Spirulina is a chemotherapeutic agent, where it causes apoptosis to tumour cells. This is shown by the reduction of the number of malignant cells and its proliferation Ki67.

Also, The ER was positive in 13/15 of DMBA group, but groups 1, 2 and 4 have similar ER immunostaining results, where ER-negative tumours are often more aggressive than the ER-positive type, making them harder to be treated successfully, so, not all DMBA-Spirulina treated group recovered but 2/15 have carcinoma in situ.

Recent studies have demonstrated that in the microalga Spirulina platensis, a blue protein called phycocyanin (PC) belonging to the photosynthetic apparatus has antioxidant and radical scavenging properties in both in vivo and in vitro models; at the same time, this natural compound is strongly anti-inflammatory (Chamorro et al., 2002). So, Using Spirulina seems to have realistic results and rapid curative effect. The development of specific therapeutic strategies based on natural algal products should be considered as an attractive approach where the conventional chemotherapy and radiotherapy have focused on mass cell killing without specific targeting and often cause damaging side effects to normal tissues.

REFERENCES
التأثير الواعي لتطبيق أسبيرولينا بلانسس على سرطان الوجبة التهويتي في الجرادان: 
دراسة مماثلة هستوكيميائية

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قسم النبات، كلية العلوم، جامعة المصورة

وتهدف البحث إلى دراسة التأثير الواقي لتطبيق أسبيرولينا بلانسس على سرطان الوجبة التهويتي في الجرادان مبكرًا داعية من أنثى أيرانيتين وعين كلا من الإستروجينات والثلاثيا الكلاسيكية Ki67 في المحاذاة النسيجية للمجموعات المختلفة باستخدام كيمياء الأنسجة المشتركة. بعد 10 يوم من عمر الجرادان تم تقسيم المجموعات كالتالي:

1. مجموعة مثبتة (1) تم إعطاؤها طبيعية.
2. مجموعة مثبتة (2) تم إعطاءها 5% طبقة أسبيرولينا إلى القدرة.
3. مجموعة (3) تم قمعها في الحيوان الطبي 35 ملجم. من قبل زيت زيتان/كم من الجرادان.
4. مجموعة (4) تم تقديمها في حبوب للكلاب 35 ملجم، زيت زيتان/كم من الجرادан و5% طبقة أسبيرولينا إلى القدرة.

تم تشفير المجموعات السابقة بعد 6 شهر من عمر الجرادان أو وصول حجم الورم الذي بين 1 - 2 سم في الوراء.

المكونات: 
• خضراوات الطماطم 
• نباتات طويلة الأجل 
• خضراوات الطماطم 
• أوراق البازلاء

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